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Misfolding of the Cystic Fibrosis Transmembrane Conductance Regulator and Disease†

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ABSTRACT: Understanding the structural basis for defects in protein function that underlie protein-based genetic diseases is the fundamental requirement for development of therapies. This situation is epitomized by the cystic fibrosis transmembrane conductance regulator (CFTR)—the gene product known to be defective in CF patients—that appears particularly susceptible to misfolding when its biogenesis is hampered by mutations at critical loci. While the primary CF-related defect in CFTR has been localized to deletion of nucleotide binding fold (NBD1) residue Phe508, an increasing number of mutations (now *ca.* 1,500) are being associated with CF disease of varying severity. Hundreds of these mutations occur in the CFTR transmembrane domain, the site of the protein's chloride channel. This report summarizes our current knowledge on how mutation-dependent misfolding of the CFTR protein is recognized on the cellular level; how specific types of mutations can contribute to the misfolding process; and describes experimental approaches to detecting and elucidating the structural consequences of CF-phenotypic mutations.

The protein molecules responsible for homeostasis are susceptible to structural perturbations induced by point mutations at "hot spots". In disease, these lead to protein misfolding, with concomitant impairment of biogenesis and/or diminution of protein function. The scope of the misfolding problem is exemplified by human genetic diseases such as cystic fibrosis (CF), where a single amino acid deletion or point mutation in one protein—the 1,480-residue cystic fibrosis transmembrane conductance regulator (CFTR)—can give a child a potentially fatal disease.

CF is a common genetic disorder in the Caucasian population, with a frequency of about one in 2500 live births. The disease affects multiple organs, presenting with chronic pulmonary obstruction, pancreatic enzyme insufficiency, elevated sweat chloride level, and reduced fertility in male patients. CFTR is normally present in the apical membrane of epithelial cells, where it functions as a chloride channel; mutations in the CFTR gene can result in defects in synthesis, trafficking, stability, function, and/or activation or regulation of the CFTR protein (*1*). A member of the ATP-binding cassette (ABC) superfamily of proteins, CFTR is organized into two transmembrane domains (TMD1 and TMD2), and two nucleotide-binding domains (NBD1 and NBD2) and a regulatory (R) domain that are cytosolic (*2*). Loss of functional CFTR at the respiratory epithelium results in altered salt concentration in the airway and dehydration of the mucus lining. CF patients thus often suffer from colonization by various bacterial species, such as *Pseudomonas aeruginosa*, in the airway, leading to chronic infection

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and inflammation (*3*, *4*). However, though the clinical consequences are often similar, the molecular basis for loss of CFTR function can differ from patient to patient. Here we review the types of intra- or interprotein interactions that most frequently cause CFTR misfolding, and describe techniques that can elucidate the structural basis of individual mutations at both the cellular and molecular levels. In tandem, these techniques provide the greatest promise for development of therapies for cystic fibrosis.

INTERACTIONS DRIVING FOLDING OF SOLUBLE AND MEMBRANE PROTEINS

Although CFTR is universally known as a "membrane protein", it contains both membrane and cytosolic (soluble) domains. Because membrane-resident and cytosol-resident sequences must be correctly incorporated into its final structure, the process of CFTR folding is complex and incorporates two sets of folding rules. In the aqueous environment of the cytosol, the hydrophobic effect drives nonpolar amino acids to the interior of the folded protein, leaving polar residues surface-exposed [*e.g.*, (*5*, *6*)]. Conversely, in the hexane-like bilayer interior where the hydrophobic effect is essentially absent, protein folding is dictated by a different set of criteria. In the latter case, folding of α-helical membrane proteins has been postulated to follow a two-stage model (*7*). In a first step, individual segments of *ca*. 18−25 residues with sufficient hydrophobic character are inserted into the membrane environment, whereupon they fold into stable transmembrane (TM) α-helices in order to minimize the free energy associated with the burial of main-chain polar groups. Protein solubility in the lipids is then facilitated via hydrophobic side chains, with Ala, Leu, Val, Ile, and Phe comprising about 80% of protein residues in membranes. In a second step, these independently folded helices then dock one with another within the membrane to adopt tertiary and/or quaternary structures stabilized by lateral, sequence-dependent helix−helix interactions.

Interactions between TM α-helices can be stabilized by van der Waals packing, where the enthalpically driven "knobs-into-holes" association becomes a primary determinant of both helix−helix contacts and the specific helix−helix interfaces produced; the close packing of TM helices is illustrated in Figure 1 for CFTR TM helices 3 and 4. A second important stabilizer of helix−helix contacts in membranes is electrostatic interactions between polar residues (categorized for discussion here as Trp, Ser, Thr, Asn, Gln, Tyr, Lys, Arg, His, Asp, Glu, and Pro). Though these side chains comprise about 15−20% of total residues native to TM domains (*8*, *9*), where they occur they can stabilize and often dominate local protein tertiary structure through the formation of side chain−side chain interhelical H-bonds within the membrane environment (*10*, *11*). In fact, nearly half of all TM helices occurring in membrane proteins solved to high resolution display a native side chain H-bond to a side chain in a neighboring helix (*12*, *13*). Other contributors to the establishment of helix−helix interfaces include cation−pi interactions (*e.g.*, between Trp and Lys residues) (*14*) and solvation effects of the lipid acyl chains surrounding the protein (*15*).

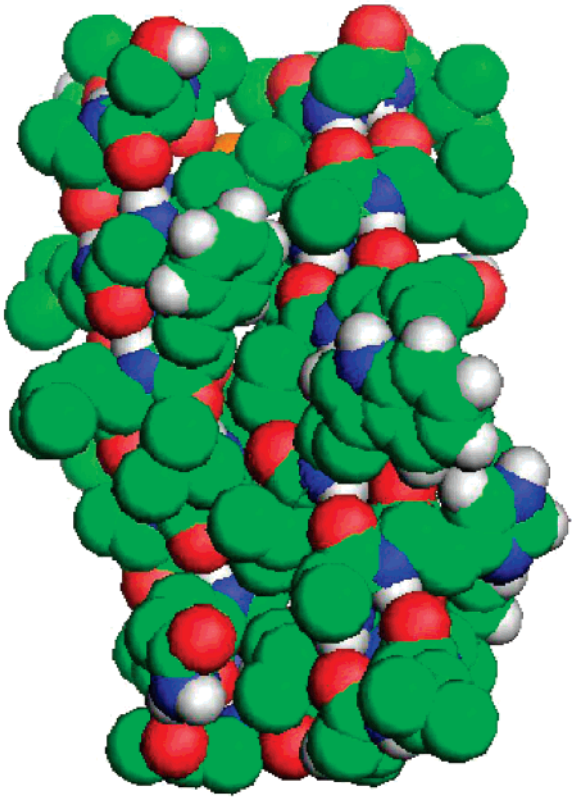

FIGURE 1: Space-filling model of CFTR transmembrane helices 3 and 4. The structure was generated using the CHI program (*86*). Residues 195−215 of TM3 (LALAHFVWIAPLQVALLMMGLI) and 221−241 of TM4 (ASAFCGLGFLIVLALFQAGLG) were used in the determination. The model displays the tight helix−helix packing between the two CFTR segments.

BIOSYNTHESIS OF CFTR: AN INTRICATE AND COMPLEX PATHWAY

Since the discovery of the gene for CF (*16*), scientists from disciplines spanning epidemiology to structural biology have been pooling their efforts to advance our understanding of this disease. The mutation ΔF508—affecting approximately two-thirds of alleles (*17*)—is the most common CFTR mutation associated with CF. This primary CF-related defect in CFTR has been localized to deletion of NBD1 residue Phe508, which causes misfolding and biosynthetic arrest of CFTR [*e.g.*, (*18*, *19*)]. Since 90% of CF patients have at least one ΔF508 allele, the molecular basis of ΔF508 CFTR pathology has emerged as the best-studied among all CFTR mutations.

In the cell, the folding pathway of CFTR begins in the lipid bilayer of the endoplasmic reticulum (ER) (Figure 2). A large number of ER-resident proteins and protein complexes are involved in the folding process of CFTR, including the translocon (reviewed in (*20*)), the oligosaccharyltransferase (*21*, *22*), and chaperones and proteins that facilitate overall folding and ER quality control (QC) (*18*). The presence of chaperones in the ER is critical for prevention of aggregation, for efficient and correct protein folding, and also for removal of misfolded proteins. For example, the ER chaperone calnexin (CNX) has a luminal lectin domain and a polypeptide-binding site which can interact with immature CFTR and aid in its folding (*23*, *24*). On the cytosolic side, CFTR interacts with Hsp70/Hsc70 and cochaperones (*25*−

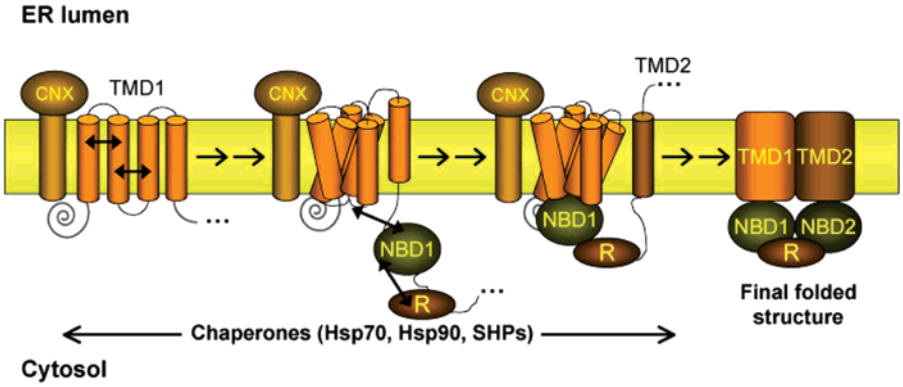

FIGURE 2: Schematic overview of the folding pathway of CFTR. The pathway likely consists of both co- and post-translational steps, and involves both intradomain (e.g., interhelical interactions in the TMDs; hydrophobic collapse in the NBDs) and interdomain interactions (e.g., TMD to NBD; TMD to TMD) to achieve the final folded structure. Examples of possible interactions are shown as double-headed arrows. Mutations in any amino acids that are crucial for these intra- or interdomain interactions ("hot spots") may disrupt the normal folding process of CFTR, resulting in kinetically trapped conformations, and ultimately compromising biogenesis or protein function. Molecular chaperones assist in this complex folding process: calnexin (CNX) mediates folding in the ER lumen by interacting with luminal loops and/or N-glycan, and perhaps assists in the assembly of the TM helices. Cytosolic chaperones (Hsp70, Hsp90, and small heat shock proteins (SHPs)) interact with cytosolic portions of CFTR.

28), Hsp90 and cochaperones (*29*, *30*), and small heat-shock protein (*31*) families.

Molecular chaperones may detect cytosolic protein misfolding by sensing the exposure of hydrophobic patches (*32*−*34*); recognition of F508 in NBD1 of CFTR is a logical example of this phenomenon. However, with respect to the TM domain, it is not clear whether misfolding can be directly detected by molecular chaperones. It is possible that CNX, which possesses a TM segment in addition to its soluble luminal lectin domain and polypeptide-binding site, and has been suggested to recognize misfolded TM domains (*35*, *36*), might maintain CFTR TM domain folding. The protein complexes involved in retrotranslocation of protein from the ER to the cytosol may also act as sensors for misfolding in the TM domain. Whatever the sensor, misfolded CFTR proteins are targeted to the ER-associated degradation (ERAD) pathway, retrotranslocated to the cytosol, ubiquitinated and degraded by the proteasome (*27*, *37*−*41*). Irrespective of mechanism, the folding of wild type (wt) CFTR is quite inefficient in heterologous expression systems, with less than 50% of the expressed protein maturing to the cell surface (*42*). However, in cells where CFTR was endogenously expressed, the maturation efficiency was nearly 100% (*43*), suggesting tissue-specific factors in these cells that help to overcome its complex folding pathway are lacking in heterologous systems.

The complexity of the CFTR folding pathway is illustrated in a number of studies. For example, the sixth TM segment in TMD1 of CFTR contains three basic amino acids and is an inefficient stop-transfer sequence that is stabilized in the membrane post-translationally by the presence of the downstream NBD1 and R-domain (*44*). Du et al. (*45*) provided evidence that full-length CFTR attains its native structure via a mechanism in which NBD1 folds co-translationally and NBD2 folds post-translationally and is affected by mutation in NBD1. One folding model proposed for CFTR suggests that individual domains fold co-translationally and immediately interact with upstream domains, and the only post-translational folding is the interaction of the two TMDs (*46*), while another found that folding of CFTR involves domain−domain interactions where TMD2, but not NBD2, is required for proper folding of the N-terminal domains and maturation of the protein (*47*). Correct folding of CFTR may also ensure trafficking of the protein from the ER to the plasma membrane by masking of ER retrieval motifs (such as arginine-framed trafficking signals) (*48*, *49*) and by exposing anterograde transport motifs (such as the diacidic motifs) (*50*), involving COPI-coated vesicles and COPII-coated vesicles, respectively.

DELETION OF F508: A MUTATION IN NBD1

As described above, the effect of deletion of F508 in CFTR presents as defective trafficking of the protein (*51*−*53*); the few ΔF508 CFTR proteins that escape the ER QC system and manage to reach the cell surface are unstable due to endocytosis and degradation (*54*, *55*). Lowering the incubation temperature of cells expressing ΔF508 CFTR allows the protein to traffic to the cell surface (*56*), leading to suggestions that the defect of ΔF508 lies in "kinetic trap(s)" in the folding pathway (*28*, *57*). Treating the cells with chemical chaperones known to stabilize protein conformation, such as glycerol, also rescues the cell surface expression of ΔF508 CFTR (*58*, *59*). Pharmacological chaperones for correcting the folding defect of ΔF508 CFTR are being investigated as potential therapies (reviewed in (*18*, *60*)).

STRUCTURAL EFFECTS OF ΔF508 IN CFTR

Consistent with the effect of the F508 deletion on CFTR stability along the folding pathway rather than as a structural defect, the recently solved crystal structures of isolated human wt NBD1 and ΔF508 NBD1 (*61*) were shown to have very similar structures, although the local surface topography at the site of mutation was altered in ΔF508 NBD1 due to a shift in position of neighboring residues G509 and V510. Proteolytic susceptibility assays also indicated that the structure of ΔF508 NBD1 was not grossly altered vs wt (*45*, *47*). As well, the isolated wt NBD1 and ΔF508 NBD1 domains do not differ significantly in terms of thermodynamic stability (*62*−*64*), consistent with crystallographic studies.

The stability of full-length ΔF508 CFTR is nevertheless altered compared to wt, as indicated by its sensitivity to

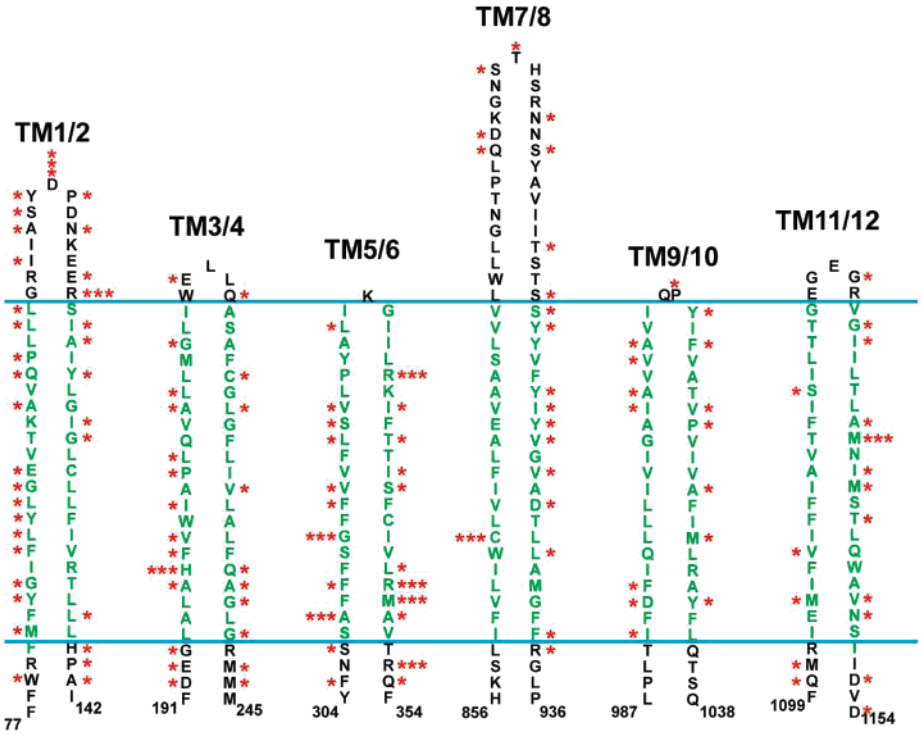

FIGURE 3: The six "helical hairpins" and inclusive extracellular loops derived from the two portions (TMD1 and TMD2) of the transmembrane domain of wild type CFTR. Residues in green are predicted to constitute the membrane-spanning TM segments of CFTR (*16*), although exact membrane entry and exit points have not been established. Blue lines delineate the bilayer cross section. Single red stars (*) indicate sites of CF-phenotypic mutations in TMD1 + TMD2 among *ca.* 625 total missense mutations reported for full-length CFTR. "Hot spots" at which three or more mutations occur are indicated (triple red stars, ***) in TMD1 at D110, R117, H199, A309, G314, R334, M335, R347, and R352; and in TMD2 at C866 and M1137. Source: CF Genetic Analysis Consortium [http://www.genet.sickkids.on.ca/cftr/].

proteolysis (*45*, *47*, *65*). F508 is located on the surface of wt NBD1, in the α-domain, which has been shown to mediate interactions between NBD and TMD in the crystal structures of three ABC transporters: BtuCD (*66*), Sav1866 (*67*), and the HI1470/1 transporter (*68*). It is conceivable that in CFTR, F508 is similarly situated at an NBD1 interface involved in a critical interaction with TMD1. Indeed, Chen et al. (*69*) showed through use of cross-linking assays that deletion of F508 in CFTR causes disruption of TMD1/TMD2 packing. Proteolysis studies have also shown that TMD1 packing is disrupted by ΔF508 (*47*). The proper integration and packing of the TMDs may therefore require NBD1 to interact properly with TMD1 (*44*).

Studies have been performed of systematic substitutions of position 508, in both isolated NBD1 and full-length CFTR. In isolated NBD1, amino acid substitutions at position 508 did not severely affect the *in vitro* folding efficiency (*64*). However, *in vivo* maturation of full-length CFTR did not tolerate substitutions at position 508 very well, further suggesting an important role for the Phe side chain in the global folding of CFTR (*37*). In general, the folding efficiency of full length CFTR was observed to be proportional to the van der Waals volume of the nonpolar side chain at position 508, with Gly being the least efficient and wild type Phe being the most efficient. Further substitutions of Phe508 with charged residues, *β*-branched nonpolar side chains, or the aromatic residues Trp and Tyr indicated that both hydrophobicity and "shape" of the amino acid side chain at position 508 may be important contributors to a compatible interface with TMD1.

CHARACTERIZATION OF CF-PHENOTYPIC MUTANTS IN CFTR

Once CFTR was recognized as the CF gene product through tracking of the ΔF508 lesion, the worldwide focus on this protein has led to an understanding that CF is not just one disease, but indeed a disorder of varying severity linked to some 1,500 mutations, as cited in the CF Genetic Consortium database [http://www.genet.sickkids.on.ca/cftr/]. These include *ca.* 625 missense mutations (*i.e.*, change of an amino acid) of which approximately 300 are localized to the 12 putative TM segments and their adjacent extracellular loops (Figure 3); the remainder are distributed throughout the cytosolic portions of CFTR. The molecular mechanism underlying CF disease varies among the wide range of CFTR mutants. Thus, the ΔF508 mutation affects primarily the trafficking of the protein, causing it to be retained in the ER and eventually degraded by the proteasome (*70*). In contrast, the mutant G551D (also located in NBD1) affects mainly the function of CFTR but not its trafficking (*71*), while the mutant G1349D, at an equivalent position in NBD2 as G551 in NBD1, similarly affects the function of CFTR (*72*). In some additional examples, a number of mutations found in the fourth intracellular loop (H1054D, G1061R, L1065P, R1066C/H/L, Q1071P, L1077P, H1085R, W1098R, M1101K/R) also affect the biosynthetic processing of CFTR (although function was not tested) (*73*); some intracellular loop 4 mutants (F1052V, K1060T, A1067T, G1069R, R1070Q/W) can process CFTR to the complex-glycosylated ("Band C") form but have altered channel activity compared to wild type. Mutants R334W, R347H/P also cause changes in ion conduction or regulation (*74*, *75*). The P67S mutation was

Table 1: Missense Mutations Arising from Single-Nucleotide Changes in the Genetic Code and in CF-Phenotypic Mutations of CFTR

Genetic Code
total number of codons: 64
% codons for polar residues = 35/64 = 55%
% codons for nonpolar residues = 26/64 = 41%
% stop codons = 3/64 = 5%
% polar aa = 12/20 = 60%
% nonpolar aa = 8/20 = 40%
no. of single-nucleotide changes possible in genetic code = 392[a]
% nonpolar → nonpolar = 90/392 = 23%
% nonpolar → polar = 74/392 = 19%
% polar → polar = 154/392 = 39%
% polar → nonpolar = 74/392 = 19%
CFTR Database of 625 Missense Mutations[b]
% nonpolar → nonpolar = 17%
% nonpolar → polar = 27%
% polar → polar = 35%
% polar → nonpolar = 21%

[a] For each one of the 64 codons, each position was changed one-by-one to each of the other three possible nucleotides. Those that produced an amino acid change were counted as one of the 392 possible changes. Nonsense mutations, silent mutations, and stop codon → aa were excluded. [b] Data taken from missense mutations listed by the CF Genetic Analysis Consortium as of mid-2007. Nonsense mutations, silent mutations, mutations that involve more than one nucleotide change, and mutations that result in no translation initiation were not included in the analysis. Some mutations may be polymorphisms not confirmed as CF-phenotypes.

found to reduce cell surface expression of CFTR but not affect the function when expressed in *Xenopus* oocytes (*76*). As further examples, the N-terminal mutations R31L/C produce CFTR molecules that are processed to Band C but have increased rates of endocytosis (*77*), while C-terminal truncation mutants can process to complex N-glycosylated forms but are subject to premature proteolysis by the proteasome (*78*).

The wealth of information available in the mutation database further indicates that a large number of CF-phenotypic missense mutations are nonconservative [*i.e.*, a nonpolar (hydrophobic) residue mutated to a polar (or charged) residue, and *vice versa*]. As mentioned above, introduction of polar mutations can influence folding by producing non-native side chain−side chain H-bonds between TM helices (*79*), or by inhibiting membrane insertion of TM helices due to reduced hydrophobicity (*80*). Since essentially all missense mutations arise from a single-nucleotide change in a given three-base codon, as noted in a survey of mutations in proteins associated with human disease (*81*), we examined the genetic code to assess its permissiveness to nonconservative mutations (Table 1). Categorizing Ala, Cys, Gly, Ile, Leu, Met, Phe, and Val as "nonpolar"—with the other 12 amino acids as "polar" (*vide infra*)—we observed that 74 of 392 possible single-nucleotide mutations (19%) change nonpolar to polar residues; examples include Gly-to-Arg, Gly-to-Glu, Val-to-Asp, Val-to-Glu, etc. Adding the reverse polar-to-nonpolar occurrences (19%), it is seen that 38% of all possible single-site missense mutations in proteins will randomly consist of nonconservative mutations. When this paradigm is applied to CFTR (Table 1), we found using the database of 625 missense mutations, that 27% involve nonpolar-to-polar changes, and another 21% involve polar-to-nonpolar changes: a total of 48% of all CF-phenotypic missense mutations. While further conclusions await an in-depth statistical analysis, a correctness-of-fit test of the data in Table 1 (ignoring a possible bias of the CFTR

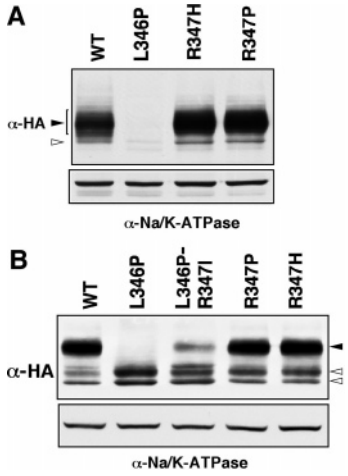

FIGURE 4: Effect of TM6 mutations on the biogenesis of CFTR. (A) Expression of wt and mutant CFTR-C_{int}HA in stably transfected BHK cells. WT, L346P, R347P, L346P/R347I, and R347H CFTR expression was assayed by immunoblotting, using the mouse monoclonal anti-HA Ab. Equal loading of proteins was verified by visualizing the Na^+/K^+-ATPase (lower panel). Empty and filled arrowheads indicate core- and complex-glycosylated forms, respectively. (B) The steady-state expression level of CFTR variants was determined in transiently transfected COS-1 cells as described in panel (A). Adapted from (*80*) and used with permission.

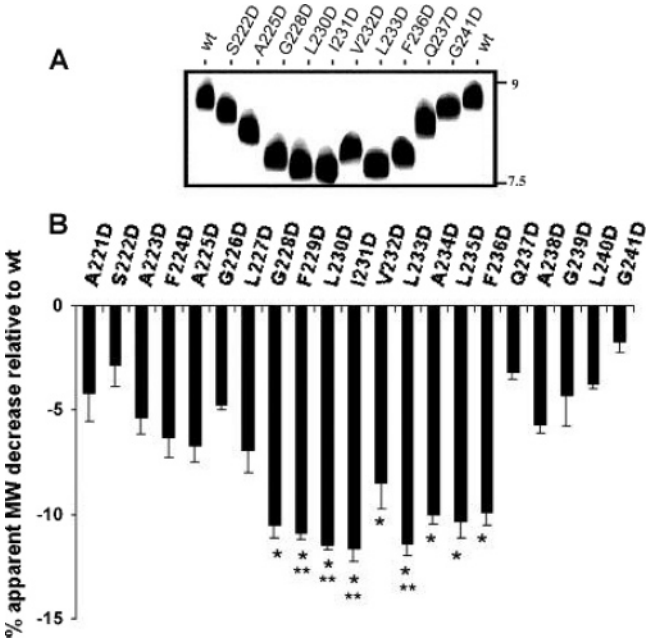

FIGURE 5: Gel shift assay for interhelical H-bonding using Western blot analysis of selected CFTR TM4 Asp mutants in CFTR TM3/4 constructs. (A) Varying migration rates indicate the relative populations of "closed" (faster migrating, strong interhelical H-bonded states) vs "open" (non-H-bonded states) of TM3/4 constructs. V232D is the CF-phenotypic mutant in TM4. Experiments are performed on 12% NuPAGE MES gels. (B) Relative apparent molecular weight % decrease for D mutants in TM4 (calculated from NIH 1.62 Image Program, software available at http://wwwcellbio.med.unc.edu/henson_mrm/pages/NIH.html). Longer downward vertical bars correspond to stronger interhelical H-bonds. Asterisks indicate varying degrees of statistically significant migration vs wild type. Adapted from (*84*) and used with permission.

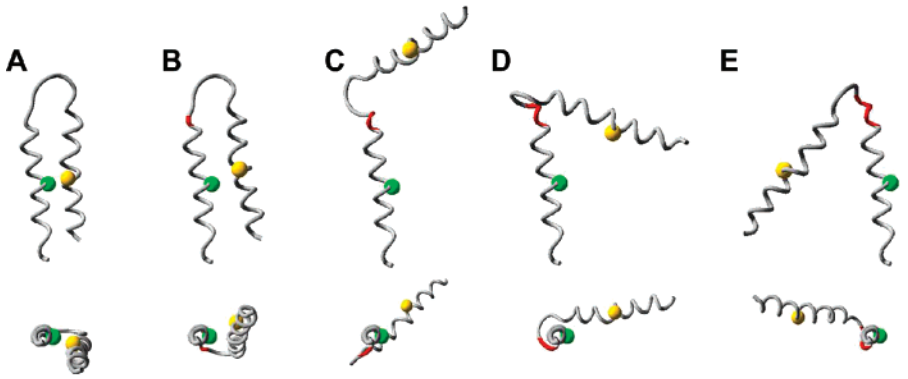

FIGURE 6: Disruption of helix−helix interactions by increased α-helical structure in the extracellular loop. The polypeptide backbone of a model helix hairpin is shown in (A) as a ribbon diagram, viewed perpendicular (top panel) and parallel (bottom panel) to the axis of the first helix. A pair of α-carbon atoms that could participate in interhelix contacts is shown in ball-and-stick representation. In (A), the backbone dihedral angles at the first 16 positions (helix 1, α-carbon in green) and final 19 positions (helix 2, α-carbon in yellow) were assigned α-helical values, and the intervening nine loop positions (gray) assigned non-regular structure. In panels B−E, successive loop positions at the end of the first helix were assigned to α-helical conformation (indicated by red shading), such that B has a single helical residue, C has two consecutive helical residues, up to four in E. The increasing α-helix structure in the loop region forces apart the TM helices, swinging helix 2 out of the plane of the page to the left, eventually placing helix 2 on the side opposite from its original position. Figure produced using SwissPDB Viewer (*87*). Adapted from (*85*) and used with permission.

total residue composition toward nonpolar residues) indicates that the difference between expected and observed values is significant at $p < 0.0001$. These data suggest that the molecular basis of protein-based genetic disease may devolve largely from disruption of protein folds in response to changes imposed by nonconservative residue mutations. For example, membrane domains of proteins may be susceptible to misfolding as a result of nonconservative mutations because introduction of a polar or charged residue in a membrane phase requires the polar side chain to minimize contact with the lipid. Patterns of phenotypic mutations occurring in the TM domains of membrane proteins linked to human disease—including CF—have been found to be dominated by mutations involving gain or loss of polar residues (*81*).

STRUCTURAL EFFECTS OF CF-PHENOTYPIC MUTATIONS ON MISFOLDING OF THE CFTR MEMBRANE DOMAIN

No high-resolution data currently exist for full-length CFTR. This reality has encouraged us to develop the use of defined domain constructs of CFTR that can be prepared in milligram amounts, manipulated readily by established biophysical techniques, and used to characterize the molecular events—such as nonconservative changes in membrane-based residues—that link CF disease to CF-phenotypic mutations. Individual TM segments have been shown to behave as independent folding domains even when excised from the protein, and to retain their native contacts (*82*). For example, TM6 in CFTR TMD1 has been implicated as central to folding pathways, as mentioned above (*36*). We therefore examined two CF-phenotypic missense mutations in the CFTR channel pore [L346P and R347P in TM6] that involve gain of a Pro residue, but where only the nonconservative mutation L346P represents a significant loss of segment hydropathy. Circular dichroism spectra of TM6 mutant peptides (not shown) revealed that L346P—but not R347P—loses *ca.* 50% helicity vs wild type, implying a membrane insertion defect in the Leu mutant (*80*). When the biogenesis of corresponding full-length CFTR mutants was examined in this context, the protein harboring the L346P mutation was found to be unstable, while the wild type, R347P, along with the R347H mutant protein, processed normally (Figure 4A). The defect could be rescued in part by restoring hydrophobicity to TM6 via the double mutant L346P/R347I (Figure 4B). Another scenario indicating the functional importance of TM-based Pro residues was presented by Thomas and co-workers, who showed that a primary role of Pro in TM segments may occur prior to their membrane insertion, via its capacity to destabilize misfolded (β-sheet aggregate) conformations (*83*).

We extended these considerations to expression in *Escherichia coli* and structural analysis of the CFTR TMD1 domain using two-TM segments (*i.e.*, helix−loop−helix constructs) which we term "helical hairpins"; these represent the minimal model of tertiary contacts between two helices in a membrane (*79*). We addressed the possibility experimentally that wild type Q207 in TM3 can form an interhelical side chain−side chain H-bond with CF-phenotypic mutant V232D in TM4 in an investigation of a series of helix−loop−helix ("hairpin") constructs derived from CFTR TM helices 3 and 4. Interhelical H-bond formation was diagnosed in SDS−PAGE gel shift assays in which the H-bond-linked hairpins forming "closed" (fistlike) conformations migrate faster than non-H-bonded "open" (rodlike) forms (Figure 5A). When this route of inquiry was extended to an "Asp-walk" over the full TM4 segment, we found that Q207 in TM3 is indeed able to "capture" all 21 TM4 D-mutations into populations of interhelical H-bonded structures varying as a function of distance of a given TM4 Asp residue from the Q207 partner in TM3 (*84*) (Figure 5B).

While the folding of membrane domains of multispanning proteins into their native functional forms clearly depends on interactions between TM helices, the covalent linker ("loop") regions may also play a fundamental role in mediating the strength of helix−helix associations. When we examined the potential structural impact of CF-phenotypic mutations in extracellular loop 2 (ECL2) (including E217G and Q220R) in a library of wild type and mutant TM3-ECL2-TM4 hairpin constructs, we found that SDS−PAGE gel migration rates differed over a range of nearly 40% +/− the wild type position, and that decreased migration rates

correlate with increasing hairpin α-helical content as measured by CD spectra in sodium dodecyl sulfate micelles (*85*). These observations were interpreted in terms of insertion of helix-promoting residues in a loop region designed to reverse chain direction (Figure 6). Although any and all effects observed in the various series' of hairpin constructs are likely to be tempered in the context of the full protein TM domain, the potential drastic structural consequences of point mutations are readily apparent.

CONCLUSION

Biogenesis and/or function of multidomain proteins such as CFTR may be severely hampered by mutations at critical loci. That human genetic disease is fundamentally a biophysical event is manifested by the observations that (i) deletion of a nonpolar side chain from the surface of a soluble domain (such as F508 from NBD1) can target the protein for swift degradation; and (ii) substitution of a native nonpolar residue with a polar residue within a membrane domain may be the underlying source of misfolding. Increased knowledge of the types of intra- or interprotein interactions that most frequently cause protein misfolding, along with development of techniques that can elucidate its structural basis and consequences at both cellular and molecular levels, provides the greatest promise for therapies. It remains an active area of research to codify how the removal or substitution of a single amino acid in a huge protein such as CFTR—and indeed in membrane proteins generally—compromises structure/function so extensively that a disease state ensues.

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